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1 Physiological and behavioural effects of exposure to environmentally relevant
2 concentrations of prednisolone during zebrafish (*Danio rerio*) embryogenesis
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Abstract

The presence of synthetic glucocorticoids within the aquatic environment has been highlighted as a potential environmental concern as they may mimic the role of endogenous glucocorticoids during vertebrate ontogeny. Prednisolone is a commonly prescribed synthetic glucocorticoid which has been repeatedly detected in the environment. This study investigated the impact of environmentally relevant concentrations of prednisolone (0.1, 1 & 10 $\mu\text{g/l}$) during zebrafish embryogenesis using physiological and behavioural endpoints which are known to be mediated by endogenous glucocorticoids. The frequency of spontaneous muscle contractions (24 hpf) was significantly reduced by prednisolone and 0.1 $\mu\text{g/l}$ increased the distance embryos swam in response to a mechanosensory stimulus (48 hpf). The percentage of embryos hatched significantly increased following prednisolone treatment (1 & 10 $\mu\text{g/l}$), while growth and mortality were unaffected. The onset of heart contraction was differentially affected by prednisolone while heart rate and oxygen consumption both increased significantly throughout embryogenesis. No substantial effect on the axial musculature was observed. Morphological changes to the lower jaw were detected at 96 hpf in response to 1 $\mu\text{g/l}$ of prednisolone. Several parameters of swim behaviour were also significantly affected. Environmentally relevant concentrations of prednisolone therefore alter early zebrafish ontogeny and significantly affect embryo behaviour.

Introduction

The presence of pharmaceuticals within the aquatic environment has emerged as a significant contributing factor to the degradation of aquatic ecosystems.^{1,2} However, the majority of toxicological studies which have attempted to assess the environmental impact of pharmaceuticals have largely overlooked synthetic glucocorticoids (SGs).^{2,3} Synthetic glucocorticoids are used in treating a variety of autoimmune and inflammatory diseases⁴ and are often excreted without being transformed into inactive metabolites³. The addition of chlorine and fluorine molecules is a common feature of SGs which increases their stability and so may also increase their longevity within the environment.⁴ Breakdown of SGs can also yield secondary compounds which are in fact more toxic than parent molecules.⁵ Considering the large quantities of these compounds which are prescribed annually⁶, and the relatively low removal rates of specific SGs from sewage management sites⁷, chronic exposure to SGs may potentially produce significant toxic effects during early development. Therefore, SGs have been highlighted as a priority for future toxicological testing.⁸

Prednisolone is the second most commonly prescribed SG in the UK⁸ and has been detected within the aquatic environment at relatively low concentrations⁹. Schriks *et al.*⁶ found concentrations of prednisolone within industrial and hospital waste water ranged between approximately 0.25 and 2 µg/l. Prednisolone has also been reported to have a relatively low removal rate from sewage treatment plants compared to other commonly occurring SGs; around 20% less.⁷ Prednisolone was first prioritised for toxicological analysis by Besse and Garric¹⁰ based upon the potential environmental risk and yet few studies have since attempted to assess its toxicity. Concentrations of prednisolone as low as 1 µg/l have been shown to significantly decrease the number of leukocytes within adult fathead minnows (*Pimephales promelas*) and significantly increase plasma glucose levels.¹¹ These changes are analogous to those effects associated with cortisol, the principal glucocorticoid in fish.¹² Synthetic glucocorticoids therefore appear to mimic the role of endogenous hormones. Synthetic glucocorticoids are potent glucocorticoid receptor (GR) agonists which allows them to directly affect the transcription and expression of specific GR mediated genes.¹³⁻¹⁵ The effects of SGs may be particularly pronounced during early ontogeny which is dependent upon endogenous glucocorticoid signalling; cortisol is known to regulate several key

physiological processes such as circadian rhythmicity¹⁶, metabolism^{17,18} and growth^{19,20} during early life stages.

Endogenous glucocorticoids play a particularly prominent role during musculoskeletal development in vertebrates and are responsible for regulating muscle mass^{21,22} and bone density²³. Sustained exposure to elevated glucocorticoid levels is associated with skeletal muscle atrophy²², an increase in the expression of myostatin, a negative regulator of muscle mass²¹ and increased bone resorption and decreased osteoblast production, leading to osteoporosis²⁴. Knockout of GR in zebrafish (*Danio rerio*) causes a delay in the formation of somites which display a misshapen phenotype and is associated with a reduction in the expression of several bone morphogenetic proteins.²⁵ The SG dexamethasone significantly alters craniofacial development during embryogenesis in zebrafish due to altered matrix metalloproteinase (MMP) expression.^{13,14}

Non-functional zebrafish GR morphants are also associated with a reduced swim activity phenotype.²⁶ Glucocorticoid mediated alterations to the development of the musculoskeletal system may therefore have a direct impact upon swimming behaviour. In addition, indirect effects may also occur. Paralysis, associated with neuromuscular disorders and muscular myopathies is connected with a reduction in the mineralisation of bone.^{27,28} Indeed, contraction of the muscle fibres is not only required for subsequent development of the muscle tissue²⁹ but is also necessary during ossification³⁰. Studies have demonstrated that swim training may accelerate the rate of ossification during development in fish.^{31,32} Therefore, the musculoskeletal system may not only be susceptible to SGs directly through developmental changes but also indirectly caused by knock on effects via alterations to individual components of the musculoskeletal system and behaviour. Consequently, analysing changes to the ontogeny of swim behaviour may offer an insightful means of assessing the impact of SGs on the musculoskeletal system.

The zebrafish embryological model offers several advantages to investigate the effects of prednisolone during early development. The onset of endogenous glucocorticoid production i.e. cortisol, has already been characterised^{33,34}, the expression of those hormones responsible for regulating cortisol production has also been studied³⁵ and GRs identified³⁶. Pikulkaew *et al.*³⁷ found that the maternally inherited *gr* transcript is the most abundant transcript inherited which encodes for nuclear and membrane steroid receptors. Glucocorticoid signalling is

therefore vital during early zebrafish development and is essential for several specific developmental processes including neural, vascular and visceral organ development.³⁷ The development of the musculoskeletal system of zebrafish has also been extensively characterised³⁸⁻⁴¹ and has been utilised as a model system to investigate muscular dystrophies in other vertebrates^{42,43}. In addition, the rapid development of the axial musculature and associated skeleton facilitates an extensive behavioural repertoire during embryogenesis in zebrafish.

Understanding the physiological effects of prednisolone exposure during early ontogeny in zebrafish may simultaneously reveal the potential environmental impact of SG pharmaceuticals within the aquatic environment, as well as contributing to our understanding of the function of endogenous glucocorticoids during vertebrate development. Therefore, the aim of this study was to explore the potential physiological and behavioural impact of exposure to environmentally relevant concentrations of prednisolone during zebrafish embryogenesis. We use Balon's⁴⁴ classification of early life stages of fishes, in which the transition from embryo to larva is defined by the onset of exogenous feeding. All experimentation was conducted using embryos.

Methods

Embryo collection

Adult zebrafish from an existing stock at the University of the West of Scotland (UWS) were maintained in 25 l glass aquaria (28±1°C; 12:12 light-dark) in a 400 l re-circulatory system which used charcoal filtered tap water. Fish were fed twice daily with flake (AQUARIAN) and once daily with *Artemia* sp. nauplii *ad libitum*. Males and females were separated prior to spawning when they were mixed within a breeding net allowing collection of embryos.

Exposure protocol

Embryos were exposed to 0.1, 1 and 10 µg/l of prednisolone (Sigma, CAS 50-24-8) which represents the upper limits of environmentally relevant concentrations of SGs⁶. Ethanol was used as a carrier solvent and stock solutions were made in 100% ethanol (Analytical grade,

Sigma, CAS 64-17-5) immediately prior to use and were kept in the dark (4°C). Water and ethanol controls (0.01% ethanol) were also tested. All embryos were continually exposed following fertilisation (from the 2-cell stage) in groups of 50 embryos using static exposures with partial renewal (50%) every 24 h. The 50% of solution which was removed daily was frozen (-20°C) for chemical analysis. A 100 ml volume of system water (water from adult fish systems) was used as an exposure medium for the majority of testing, however, for the purposes of improving survivability into later stages of development, groups of embryos (including controls) which were used to investigate musculoskeletal and behavioural parameters were exposed to prednisolone from the 2-cell stage in 50 ml of embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM MgSO₄·7H₂O, 0.33 mM CaCl₂·2H₂O and 10⁻⁵% of methylene blue in distilled water)⁴⁵. All embryos were maintained at 28±1°C using water baths and any embryos which failed to develop normally were removed daily and mortality was recorded.

In the event that dechorination was required to measure specific endpoints before hatching had occurred, only dechorinated embryos were used. Similarly, in the event that freely hatched embryos were required, only freely hatched embryos were used. In both cases, this ensured comparisons between treatments were made on individuals which were at the same stage of development. Replication varied between the individual parameters measured and is therefore stated in the relevant section of the methods, however, a minimum of three beaker replicates of each experimental treatment were tested for all parameters.

Chemical analysis

Water samples were analysed at the University of Santiago de Compostela (USC), Spain via liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a previously developed method for analysing pharmaceuticals including corticosteroids⁴⁶. Water samples (25 ml) were filtered under vacuum using a 0.47-µm glass microfiber filter, from Filter-Lab (La Rioja, ES). Filtered samples were acidified with 0.1 N-HCl solution to a pH of 3 and 10 µl of the IS solution (1 µg/ml) were added. The samples were shaken and transferred into Strata®-X SPE cartridges that were conditioned with 4 ml of methanol (CAS 67-56-1) and 4 ml of Milli-Q water. The samples were loaded into the cartridges at a flow rate of 1 ml/min. Cartridges were dried under vacuum for 30 min. The sample vessels were rinsed with 4 ml of methanol which was transferred to the SPE cartridges and was left to soak for 5 min; the

eluent was collected in a 10 ml conical graduated glass Pyrex® tube (16.5×110 mm) with 4 ml additional methanol. The final extract was evaporated under nitrogen at 45°C. The dried extracts were reconstituted with 200 µl of 0.1% formic acid (CAS 64-18-1) in methanol, vortexed and transferred into a 300 µl glass insert and fused into an amber 2 ml screw top standard vial (12×32 mm). The extracts were stored at -20°C until further analysis by LC-MS/MS.

Samples were analysed on a LC-MS/MS system consisting of an HPLC 1100 separation module from Agilent Technologies (Waldbronn, Germany), a Qtrap 2000™ mass spectrometer from Applied Biosystems/MDS Sciex (Toronto, Canada) equipped with a TurboIonSpray® source, and the Software Analyst 1.4.1 from Applied Biosystems. The chromatographic analyses were performed by injecting 10 µl of extract into a Synergi 2.5 µm Polar-RP 100A column (50×2.0 mm) connected to a Polar-RP security-guard cartridge (4.0×2.0 mm), both obtained from Phenomenex (Macclesfield, UK). Separation of analytes was achieved using a gradient mixture of two components, 0.1% formic acid in acetonitrile (CAS 75-05-8) and 0.1% formic acid in water. The gradient programme is reported in Iglesias *et al.*⁴⁶

Mass-spectrometry (MS) measurements were performed using positive electrospray, and drug identification was performed using two multiple reaction monitoring transitions and their retention times. The following MS parameters were utilised for prednisolone measurements: declustering potential, 21; entrance potential, 6; collision cell entrance potential, 14; collision energy, 29 and cell exit potential, 4. A source temperature of 400°C, a vacuum gauge of 22×10^4 Pa, an ion spray voltage of 5.5 V and a curtain gas pressure of 17×10^4 Pa were maintained throughout. The first ion source was set at 38×10^4 Pa, and the second ion source at 34×10^4 Pa. For all monitored transitions, the dwell time was 10 ms.

The limits of detection and quantification were 12.5 and 25 ng/l. Preliminary testing found that samples collected at 0 h were 0.1 ± 0.05 , 1.1 ± 0.63 and 8.2 ± 0.45 µg/l. No prednisolone was detected within either control group. Unfortunately, the majority of samples collected throughout the experiment defrosted in transit between UWS and USC which caused degradation of the samples. Results are therefore discussed in terms of nominal concentrations of prednisolone at the start of exposure.

Pre-hatch activity

The onset of pre-hatch activity was monitored at 15, 16 and 17 hours post fertilisation (hpf). Embryos were visualised in the chorion within the beakers in which they were maintained under a dissection microscope. Care was taken not to cause any sudden vibrations along the bench which could cause a startle response during observation. Embryos were observed for 1 minute after an initial 1 minute acclimation period. The percentage of embryos which displayed stereotypic body contractions at each time point was recorded. Four beaker replicates of each experimental treatment were visualised ($n = 4$). The frequency of spontaneous contractions at 24 hpf was also recorded using four embryos from each of the four treatment replicates ($n = 16$). Embryos were observed for 3 minutes after an initial 1 minute acclimation period and the mean number of contractions was calculated per minute (contractions per minute, cpm).

Touch evoked escape response

Methods were adapted from Smith *et al.*⁴⁷. Individual freely-hatched embryos (48 hpf) were placed into a 6 cm diameter petri dish filled with clean embryo media at 28°C and were viewed under a JVC TH-C1480B colour camera connected to a desktop PC. Embryos were centred under the field of view and were filmed from prior to the onset of the mechanosensory stimulus. The mechanosensory stimulus was applied to the tail of the embryo using a blunt needle tip. Imaging ceased once the embryo had ceased swimming. Still images were taken throughout the touch response (every 25 frames), including the initial and final positions of the embryo. Images were then analysed using ImageJ⁴⁸ to calculate distance travelled between consecutive images. Total distance travelled (mm) was then calculated. Four embryos from each of three treatment replicates were tested ($n = 12$).

Hatching & growth

Four replicates of each experimental treatment were screened at 24 h intervals to record the number of embryos hatched over the first 96 h of development ($n = 4$). Length and yolk-sac area was analysed as an indicator of growth at 24, 48 and 72 hpf. Four embryos from each of four treatment replicates were terminally anaesthetised using buffered MS-222 (0.08%).

Images of the embryos were taken using a Leica MZFIII photo-microscope and Leica Image Manager 50 and viewed using ImageJ⁴⁸. The length of embryos, the distance from the most anterior part of the head to the tip of the tail following the path of the developing spinal cord⁴⁹, and the area of the yolk-sac (including the yolk-extension) were measured ($n = 16$) at each time point. Embryos were dechorionated at 24 hpf to facilitate photography and morphometric analysis while freely-hatched embryos were used at 48 and 72 hpf.

Cardiac development

Single embryos (20 hpf) were placed into individual wells of a 96 well plate filled with 360 μ l of the appropriate concentration of prednisolone or control media. The entire plate was covered to prevent evaporation while it was maintained in an incubator at 25°C. Embryos were viewed at 1 h intervals over the course of 4 h and the percentage of embryos which displayed a visible heart beat was recorded. Maintaining embryos at 25°C reduced the rate of development and enabled any effect of prednisolone on the onset of heart beat to be highlighted. Four embryos from each of the six treatment replicates were used to calculate the percentage of embryos with a visible heart beat ($n = 6$). Heart rate was recorded at 24, 48 and 72 hpf at 28°C. Individual embryos were visualised using a digital EUROMEX 6.0 M Pixel camera attached to a NOVEX Holland B-series microscope connected to a desktop PC. Embryos at 24 hpf were visualised within the chorion. At 48 and 72 hpf, freely-hatched embryos were mounted in a 6% methylcellulose solution on a microscope slide to maintain their position during recording. A drop of water was added on top of the methylcellulose to prevent desiccation during observation. Embryos were left to acclimate within the methylcellulose for 15 minutes prior to visualisation on top of a microscope slide heater to provide a constant temperature. An additional 1 minute acclimation period was provided while positioned on the microscope stage. Heart rate in beats per minute (bpm) was calculated as the mean number of beats during three 20 s periods, multiplied by three. Heart rate was measured using four embryos from three replicates of each treatment ($n = 12$).

Metabolism

Oxygen consumption was used as an indicator of metabolic rate and was measured using methods modified from Sloman¹⁸ at 24, 48 and 72 hpf. Oxygen consumption was measured using sealed respiratory chambers consisting of 20 ml blackened glass vials containing a

magnetic stirrer separated from the main body of the respirometer by a horizontal mesh partition. Magnetic fleas prevented an oxygen gradient forming within the chamber. Each respiratory chamber contained three embryos from the same replicate, and two respiratory chambers were tested for each of four treatment replicates ($n = 8$). Chambers were filled with system water and were maintained at $28 \pm 1^\circ\text{C}$ in water baths. Water samples (0.1 ml) were taken from each chamber after a 4 h period and were immediately injected into a thermostatted cell (Strathkelvin Instruments, Glasgow, UK), containing an oxygen electrode connected to an oxygen meter. Embryos were then removed from the chambers and weighed on a balance. Calibration of the oxygen electrode used air-saturated water from the water baths as 100% saturation and a 2% solution of sodium sulphite (in 0.01 M sodium borate) as a 0% standard. The oxygen saturation values were then converted into μmol of oxygen which were adjusted for temperature and air-pressure and used to calculate mass specific oxygen consumption. Blank chambers served as controls to account for bacterial oxygen consumption.

Skeletal muscle development

Morphometric measurements were taken at 72 hpf. Anaesthetised embryos (0.04% MS-222) were visualised laterally under a dissection microscope attached to a EUROMEX 6.0 M Pixel camera connected to a desktop PC. The area of the anal (AN), pre (PA) and post-anal (PSA) somites (Figure 1A) was calculated in addition to the angle connecting these segments. Six embryos from three treatment replicates were photographed ($n = 18$). Muscle integrity was visualised *via* birefringence using methods adapted from Smith *et al.*⁴⁷. Polarised lens filters were positioned between the embryo and the microscope lens. The upper filter was then rotated to align both filters at 90° to eliminate any light which was not perpendicular to the muscle fibres (Figure 1B). The mean intensity of the skeletal muscle was divided by the area of muscle, and then normalized to control values. Four embryos were photographed from each of four treatment replicates ($n = 16$). All images were processed using Image J.

Craniofacial morphogenesis

Developmental changes to the anterior skeleton of embryos were investigated at 96 hpf. Embryos were euthanatized using an overdose of MS-222 (0.08%), fixed overnight at 4°C in 4% phosphate-buffered formaldehyde, washed in PBS with 0.1% Tween-20 (PBT) and then

bleached in 3% hydrogen peroxide/ 1% potassium hydroxide. Embryos were then rinsed with PBT and transferred into 0.1% alcian blue solution (1% concentrated hydrochloric acid, 70% ethanol) and stained overnight. After rinsing several times with acidic ethanol (5% concentrated hydrochloric acid, 70% ethanol) embryos were rehydrated through an HCl-EtOH series (75%, 50%, 25% and 0%) and stored in glycerol. Embryos were then photographed ventrally to capture the anterior portion of the developing skeleton using a EUROMEX 6.0 M Pixel camera (Figure 1C). Criteria for assessing skeletal development were adopted from Hillegass *et al.*¹⁴. The intercranial distance (ICD), lower jaw length (LJL), and ceratohyal cartilage length (CCL) were measured using ImageJ. Four embryos were photographed from each of four treatment replicates ($n = 16$).

Swimming behaviour

Swimming behaviour of individual embryos was assessed at 120 hpf in groups of five embryos which were placed into a 6 cm diameter petri dish marked with a grid floor (4.5 mm²; approx. one body length) filled with clean embryo media (28°C). After an initial 2 minute acclimation period within the dish, activity was recorded for 10 minutes. Behaviour was recorded using a JVC TH-C1480B colour camera positioned above the test chamber. The percentage of time spent swimming, distance swum (no. of body lengths swum; bls) and the average swim speed (body lengths per second; blsps) was calculated. Five embryos were filmed from each of four treatment replicates ($n = 20$).

Statistical analysis

All statistical analysis was conducted using SPSS v.18. Data were checked for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively. Where data were not normally distributed, or in the case of percentage data, transformations were conducted to allow parametric testing. No significant effect of replicate was found and so data were combined within treatments. Two-way ANOVAs were used to determine the effects of treatment and time on all measurements. Where significant interactions occurred within the ANOVA model, time and treatment effects were further analysed using one-way ANOVAs with Fisher's LSD test post hoc. Non-parametric equivalents were used where necessary (Scheirer-Ray-Hare (SRH), Kruskal- Wallis test (KW) and pairwise Mann-Whitney U).

Results

Pre-hatch activity

There was no significant difference in the onset of spontaneous contractions in response to prednisolone in pre-hatched embryos, however, all concentrations tested significantly reduced the frequency of spontaneous contractions at 24 hpf (Table 1, Figure 2A).

Touch evoked escape response

The touch-evoked escape response of freely-hatched embryos at 48 hpf increased following treatment with 0.1 µg/l of prednisolone (Table 1, Figure 2B).

Hatching & growth

Exposure to 1 and 10 µg/l prednisolone induced precocious hatching at 48 and 72 hpf (Table 1, Figure 3), although prednisolone did not significantly alter length or yolk-sac area during embryogenesis. Mortality did not vary during the first 24 hours of development between treatments and no mortality was observed after this period.

Cardiac development

A significantly higher proportion of embryos exhibited a visible heart beat following 0.1 µg/l of prednisolone at 23 hpf, while 10 µg/l of prednisolone significantly reduced the onset of heart beat at 23 and 24 hpf (Table 1, Figure 4A). In contrast, heart rate was significantly increased by all concentrations of prednisolone between 24 and 72 hpf (Table 1, Figure 4B).

Metabolism

The rate of oxygen consumption between 24 and 72 hpf was significantly increased by all concentrations of prednisolone but at specific time points (Table 1, Figure 5).

Skeletal muscle development

The area of pre-anal somites were reduced in response to 0.1 µg/l of prednisolone, without affecting the angle of attachment between somites or the integrity of skeletal muscle (Table 1).

Craniofacial morphogenesis

There was also an overall enlargement of the cranial skeleton following 1.0 µg/l of prednisolone at 96 hpf, as all indices of craniofacial morphogenesis were significantly increased (Table 1, Figure 6).

Swimming behaviour

Finally, prednisolone significantly reduced the percentage of time embryos spent swimming (10 µg/l), the distance swum (0.1, 1.0 and 10 µg/l) and the average swim speed (1.0 and 10.0 µg/l) at 120 hpf (Table 1, Figure 7).

Discussion

Exposure to environmental concentrations of prednisolone during embryogenesis resulted in significant physiological and behavioural effects throughout ontogeny. While the onset of spontaneous muscular contractions did not change in response to prednisolone treatment, the frequency of contractions were significantly reduced (Figure 2A). Exposure to 0.1 µg/l of prednisolone also increased the touch evoked escape response to a mechanosensory stimuli at 48 hpf (Figure 2B). No effect of the higher concentrations of prednisolone on the escape response was observed and it may be prudent to interpret the fact that an effect was only seen at the lowest dose with caution. Regardless, zebrafish embryos which exhibit defects in skeletal muscle development are typically associated with a diminished escape response⁴⁷ which suggests that the developing musculature has not been negatively affected by prednisolone at this stage. Spontaneous contractions, as well as the startle response, are regulated by the neural circuitry within the spinal cord⁵⁰⁻⁵³ and the Mauthner neurons in particular are associated with regulating the escape response following mechanosensory stimulus⁵⁴. Whether the neural circuitry has been altered in response to prednisolone however requires specific investigation.

The increase in the percentage of embryos hatched following prednisolone treatment (Figure 3) agrees with Wilson *et al.*⁵⁵ who found that exposure to dexamethasone also resulted in precocious hatching in zebrafish embryos. Hatching is facilitated by a combination of

enzymatic digestion of the chorion and mechanical damage due to embryonic activity.⁵⁶ Since the frequency of pre-hatch activity significantly decreased, the increased hatching is more likely to be related to changes in the release of proteolytic enzymes. Cloud⁵⁷ also hypothesised that exposure to exogenous steroids led to changes in the release of hatching enzymes. No statistically significant change in either length or yolk-sac area was found which suggests that growth was not detrimentally affected by prednisolone. This is in contrast to previous studies have shown diminished growth rates and reduced yolk-sac volumes in response to elevated glucocorticoid exposure.^{19,20}

The onset of heart contraction was significantly altered by prednisolone (Figure 4A). While the heart is the first organ to fully develop during vertebrate embryogenesis⁵⁸, embryonic and early larval stages do not require a functional cardiovascular system for oxygen delivery⁵⁹. The “prosynchrotropy” hypothesis proposes that the early onset of cardiovascular contraction is primarily related to cardiac morphogenesis⁶⁰, which has been demonstrated experimentally by Hove *et al.*⁶¹. An earlier onset of heart contraction in response to 0.1 µg/l of prednisolone could therefore suggest an overall acceleration of cardiac development. However, exposure to 10 µg/l prednisolone also produced a delay in the onset of heart contraction. Biphasic physiological responses to xenobiotics are not uncommon although the precise underlying mechanisms are not clear, especially since both concentrations increased heart rate (Figure 4B). This could suggest that the developmental processes which regulate cardiogenesis and the physiological mechanisms which control heart rate are differentially regulated by glucocorticoids. Glucocorticoid signalling during early zebrafish ontogeny has been recently shown to significantly affect the structure and function of the heart in embryos.⁶² In addition, exposure to dexamethasone (100 µM) increases the size of embryonic hearts and enhances cardiac performance. Interestingly, injection of cortisol into zebrafish embryos causes heart deformities and suppression of essential cardiac genes.⁶³ Wilson *et al.*⁶² also found that zebrafish GR knockouts displayed diminished heart maturation. The intensity of glucocorticoid signalling therefore appears to differentially alter heart development and further study is necessary to elucidate the precise mechanisms of glucocorticoid mediated effects on cardiac development.

Embryos exposed to prednisolone consumed more oxygen throughout the first 72 hours of development (Figure 5). This is in agreement with findings reported by Giesing *et al.*¹⁷ and Sloman¹⁸ and who found that exposure to endogenous glucocorticoids increased oxygen

consumption during embryonic development in stickleback (*Gasterosteus aculeatus*) and brown trout (*Salmo trutta*) respectively. The increase in oxygen consumption may be related to an increase in metabolic rate as glucocorticoids are essential regulators of metabolism in teleosts.⁶⁴ Metabolic enzymes are sensitive to SG exposure during early development in zebrafish, e.g. phosphoenolpyruvate carboxykinase, which controls the rate limiting step in gluconeogenesis, increases in response to dexamethasone.⁶⁵ Similarly, prednisolone (1 µg/l) is known to increase plasma glucose concentrations in adult fathead minnow.¹¹ Increased metabolic rate could also accelerate organogenesis⁶⁶ which is prioritised over growth during ontogeny⁶⁷ and would facilitate an increase in hatching and heart maturation.

Although there was an almost 10% reduction in birefringence in response to 10 µg/l of prednisolone, in a manner consistent with a myopathic phenotype⁴⁷, no statistically significant difference between treatments was found. Nor were there any significant changes in the angle of attachment between adjacent somites. However, exposure to 0.1 µg/l of prednisolone resulted in significantly smaller pre-anal somites at 72 hpf. Given the relatively small change in the area of somites between treatments (approx. 5%), and no observable change in muscle integrity, the significance of these changes are likely to be minor since changes were localised to individual somites at specific concentrations of prednisolone. Swimbladder inflation was also unaffected by prednisolone treatment (data not shown) which suggests that this small change in somite size did not affect swim-up behaviour and is therefore likely to be physiologically insignificant.

Craniofacial morphogenesis was significantly altered in response to 1 µg/l of prednisolone at 96 hpf (Figure 6). Intercranial distance, LJL and CCL all increased, which suggests an overall enlargement of the cranial skeleton. Prednisolone has previously been shown to induce bone loss in zebrafish embryos but at far greater concentrations than those utilised in this study.⁶⁸ Dexamethasone also significantly alters craniofacial development in zebrafish embryos by altering the expression of MMPs which culminates in significantly longer heads.^{13,14} Whether prednisolone may also affect axial skeletal development requires additional investigation, however, it is unlikely that the increase in skull size led to the observed changes in swim behaviour, since 0.1 µg/l and 10 µg/l of prednisolone also caused behavioural effects, despite failing to alter skeletal development.

Embryos at 120 hpf exhibited significantly reduced swim activity in response to prednisolone treatment (Figure 7). This coincides with behavioural changes in response to dexamethasone exposure⁶⁹ and from studies which have prevented normal glucocorticoid signalling during zebrafish development^{26,55}. Reductions in spontaneous activity during pre-hatch and free-swimming stages could suggest common physiological mechanisms may be susceptible to prednisolone throughout development. Alternatively, changes during early ontogeny may persist into free swimming stages. It may also be possible that higher concentrations of prednisolone may affect additional physiological systems which regulate behaviour; increasing concentrations of prednisolone produced differences in the swim profile of free swimming embryos.

Given the parity between these findings and those from previous studies which have investigated glucocorticoid signalling during zebrafish ontogeny, we tentatively hypothesis that prednisolone toxicity may be primarily mediated through binding to the glucocorticoid receptor and subsequent modulation of glucocorticoid regulated pathways. Glucocorticoid signalling is responsible for regulating hundreds of genes; knockout of the maternally derived *gr* mRNA results in several developmental abnormalities within neural, vascular and visceral tissues.³⁷ Exposure to SGs during early development may similarly interfere with multiple processes and would therefore suggest multiple molecular pathways may be responsible for the observed developmental changes. However, commonality with such studies may point to disruption to the hypothalamic-pituitary-interrenal (HPI) axis, which controls the release of cortisol and thereby regulates homeostasis¹², as a logical starting point for future investigation. Cortisol is known to mediate several essential physiological processes such as metabolism and growth during early life stages. In addition, the persistent behavioural changes throughout development observed in this study also bear semblance to how changes in cortisol levels affect the neurological programming of individuals and subsequent behavioural phenotypes.^{70,71} Glucocorticoids are believed to serve an active role during neurogenesis^{70,71} and could have subsequent effects on brain monoamines⁷² which are important neural regulators of behavioural phenotypes in zebrafish⁷³.

The results from this study (summarised in Table 2) contribute to the growing body of evidence that SGs can alter several physiological processes during early development in fish, in a manner consistent with the effects of endogenous glucocorticoid signalling. Such

changes may have significant consequences upon the future development, performance and survival of zebrafish. Further investigations are therefore required to identify the underlying mechanisms for these changes during development and the potential long term implications also need to be clarified.

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Table legends

Table 1: Summary of statistical analysis for all parameters measured. P values in bold indicate significant effects.

Table 2: Summary of significant developmental effects following prednisolone treatment. ↓ indicates statistically significant decrease compared to controls; ↑, significant increase compared to controls; -, no significant change. ICD, intercranial distance; LJL, lower jaw length; CCL, ceratohyal length; hpf, hours post fertilisation.

Figure legends

Figure 1: (A) Control embryo (72 hpf) showing somites analysed for morphometric analysis, pre-anal (PA), anal (AN) and post-anal (PSA). (B) Control embryo (72 hpf) under polarized light. Scale bars = 0.5 mm. (C) Control embryo at 96 hpf stained with 0.1% alcian blue. Numbers correspond with defined parameters; 1, lower jaw length (LJL); 2, intercranial distance (ICD); 3, ceratohyal cartilage length (mm). Parameters 1 and 3 begin from the dashed line. Scale bar = 250 μ m.

Figure 2: (A) Mean number of spontaneous contractions per minute at 24 hpf ($n = 16$). Letters denote significant differences between treatments at specific time points ($P < 0.05$). Where bars share letters there is no significant difference. Means \pm SEM. (B) Distance travelled during touch invoked escape response at 48 hpf ($n = 12$). Letters denote significant differences between treatments ($P < 0.05$). Means \pm SEM.

Figure 3: The percentage of hatched embryos over the first 96 h of development ($n = 4$). Letters denote significant differences between treatments at specific time points ($P < 0.05$). Where bars share letters there is no significant difference. Means \pm SEM.

Figure 4: (A) The percentage of embryos which displayed a visible heart beat during the first 25 h of development in response to prednisolone ($n = 6$). (B) Heart rate (beats per minute, bpm) of embryos at 24, 48 and 72 hpf in response to prednisolone ($n = 16$). Letters denote significant differences between treatments at specific time points ($P < 0.05$). Where bars share letters there is no significant difference. Means \pm SEM.

Figure 5: The oxygen consumption (μ mol.g⁻¹h⁻¹) of embryos at 24, 48 and 72 hpf in response to prednisolone ($n = 8$). Letters denote significant differences between treatments at specific time points ($P < 0.05$). Where bars share letters there is no significant difference. Means \pm SEM.

Figure 6: Craniofacial measurements (mm) at 96 hpf ($n = 16$). Letters denote significant differences between treatments at specific time points ($P < 0.05$). Where bars share letters there is no significant difference. Means \pm SEM.

Figure 7: (A) Percentage of time swimming at 120 hpf ($n = 20$). (B) Activity (number of body lengths swum) at 120 hpf ($n = 20$). (C) Average swim speed at 120 hpf ($n = 20$). Letters denote significant differences between treatments ($P < 0.05$). Means \pm SEM.

<i>Developmental endpoints</i>		<i>Test</i>	<i>Test statistic</i>	<i>P-value</i>
<i>Pre-hatch activity</i>	<i>Onset</i>	<i>ANOVA</i>	$F_{4,19} = 0.424$	$P = 0.789$
	<i>Frequency</i>	<i>ANOVA</i>	$F_{4,79} = 7.268$	$P < 0.001$
<i>Touch evoked escape response</i>		<i>ANOVA</i>	$F_{4,56} = 3.437$	$P = 0.016$
<i>Hatching & growth</i>	<i>Hatching</i>	<i>2-Way ANOVA</i>	Time, $F_{1,39} = 280.154$	$P < 0.001$
			Treatment, $F_{4,119} = 13.206$	$P < 0.001$
			Time*Treatment, $F_{4,39} = 4.279$	$P = 0.007$
	<i>Length</i>	<i>2-Way ANOVA</i>	Treatment, $F_{4,230} = 1.865$	$P = 0.118$
			Time, $F_{2,230} = 174.667$	$P < 0.001$
			Treatment*Time, $F_{8,230} = 0.822$	$P = 0.584$
<i>Cardiac development</i>	<i>Onset of heart beat</i>	<i>2-Way ANOVA</i>	Treatment, $F_{4,231} = 8.574$	$P = 0.373$
			Time, $F_{2,231} = 89.84$	$P < 0.001$
			Treatment*Time, $F_{8,231} = 4.577$	$P = 0.805$
	<i>Heart rate</i>	<i>2-Way ANOVA</i>	Time, $F_{1,60} = 82.147$	$P < 0.001$
			Treatment, $F_{4,60} = 11.532$	$P < 0.001$
			Time*Treatment, $F_{4,60} = 4.557$	$P = 0.003$
<i>Metabolism</i>	<i>Oxygen consumption</i>	<i>2-Way ANOVA</i>	Time, $F_{1,119} = 527.366$	$P < 0.001$
			Treatment, $F_{4,119} = 26.055$	$P < 0.001$
			Time*Treatment, $F_{4,119} = 2.571$	$P = 0.042$
<i>Skeletal muscle development</i>	<i>Area of somites</i>	<i>ANOVA</i>	Time, $F_{2,119} = 30.586$	$P < 0.001$
			Treatment, $F_{4,119} = 8.667$	$P < 0.001$
			Time*Treatment, $F_{8,119} = 8.667$	$P < 0.001$
	<i>Angle of attachment</i>	<i>ANOVA</i>	Pre-anal, $F_{4,89} = 2.547$	$P = 0.045$
			Anal, $F_{4,89} = 1.148$	$P = 0.340$
			Post-anal, $F_{4,89} = 2.954$	$P = 0.024$
<i>Craniofacial morphogenesis</i>	<i>Muscle integrity</i>	<i>ANOVA</i>	Pre-anal, $F_{4,89} = 1.047$	$P = 0.388$
			Anal, $F_{4,89} = 1.284$	$P = 0.283$
			Post-anal, $F_{4,89} = 1.747$	$P = 0.147$
<i>Swimming behaviour</i>	<i>Area of somites</i>	<i>ANOVA</i>	$F_{4,74} = 0.363$	$P = 0.834$
			Area, $F_{2,206} = 817.249$	$P < 0.001$
			Treatment, $F_{4,206} = 10.098$	$P < 0.001$
<i>Swimming behaviour</i>	<i>Time swimming</i>	<i>Kruskal-Wallis</i>	Area*Treatment, $F_{8,206} = 0.606$	$P = 0.772$
			$\chi^2 (4) = 13.344$	$P = 0.01$
<i>Swimming behaviour</i>	<i>Distance swum</i>	<i>ANOVA</i>	$F_{4,97} = 16.258$	$P < 0.001$
	<i>Swim speed</i>	<i>ANOVA</i>	$F_{4,97} = 2.682$	$P = 0.036$

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				<i>Concentration of prednisolone (µg/l)</i>		
<i>Developmental endpoints</i>		<i>Stage of development (hpf)</i>		<i>0.1</i>	<i>1.0</i>	<i>10.0</i>
<i>Pre-hatch activity</i>	<i>Onset</i>		15, 16, 17	-, -, -	-, -, -	-, -, -
	<i>Frequency</i>		24	↓	↓	↓
<i>Touch evoked escape response</i>			48	↑	-	-
<i>Hatching & growth</i>	<i>Hatching</i>		48, 72, 96	-, -, -	-, ↑, -	-, ↑, -
	<i>Length</i>		24, 48, 72	-, -, -	-, -, -,	-, -, -,
	<i>Yolk-sac area</i>		24, 48, 72	-, -, -	-, -, -,	-, -, -,
<i>Cardiac development</i>	<i>Onset of heart beat</i>		23, 24, 25	↑, -, -	-, -, -	↓, ↓, -
	<i>Heart rate</i>		24, 48, 72	↑, ↑, -	↑, ↑, ↑	↑, ↑, ↑
<i>Metabolism</i>	<i>Oxygen consumption</i>		24, 48, 72	-, ↑, -	↑, ↑, -	↑, ↑, ↑
<i>Skeletal muscle development</i>	<i>Area of somites</i>	<i>Pre-anal</i>	72	↓	-	-
		<i>Anal</i>	72	-	-	-
		<i>Post-anal</i>	72	-	-	-
	<i>Angle of attachment</i>		72	-	-	-
	<i>Muscle integrity</i>		72	-	-	-
<i>Craniofacial morphogenesis</i>	<i>ICD</i>		96	-	↑	-
	<i>LJL</i>		96	-	↑	-
	<i>CCL</i>		96	-	↑	-
<i>Swimming behaviour</i>	<i>Time swimming</i>		120	-	-	↓
	<i>Distance swum</i>		120	↓	↓	↓
	<i>Swim speed</i>		120	-	↓	↓

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Figures

Figure 1

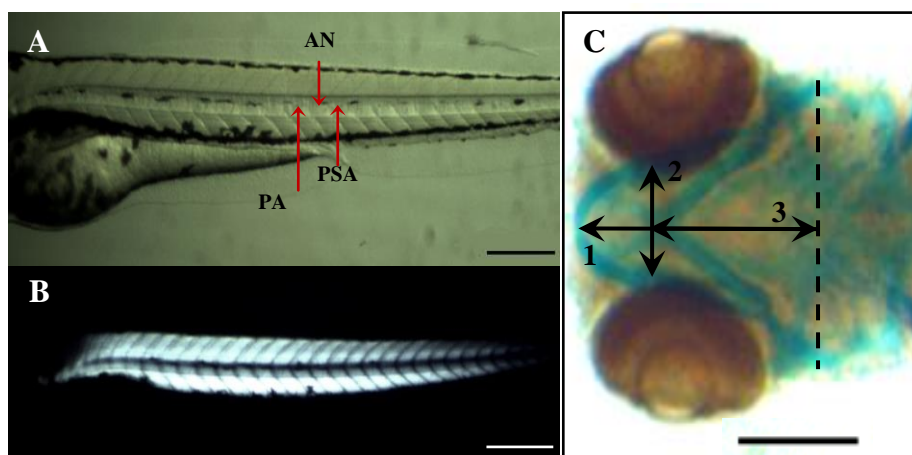


Figure 2A

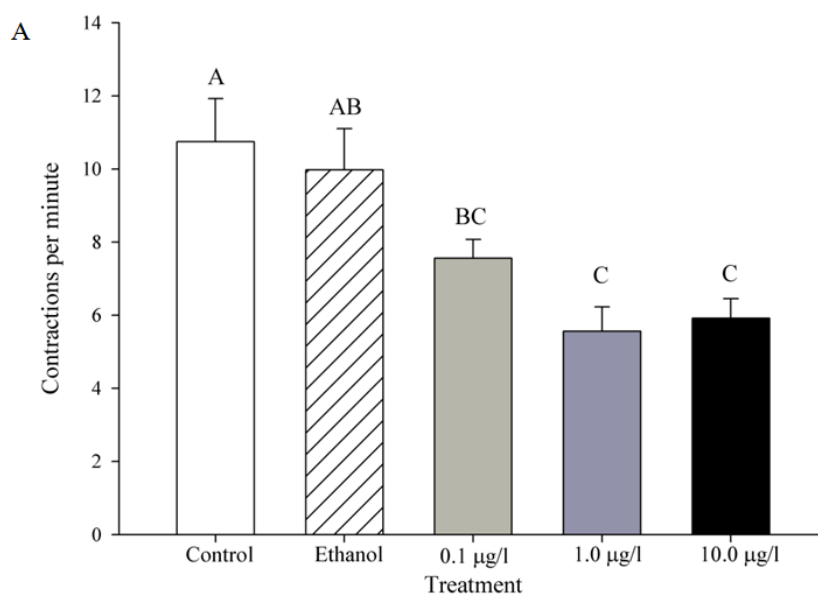


Figure 2B

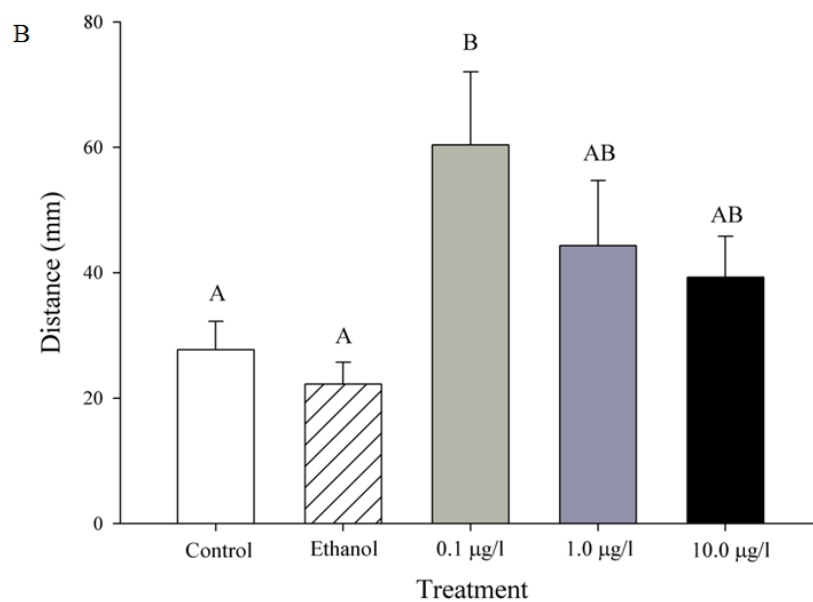


Figure 3

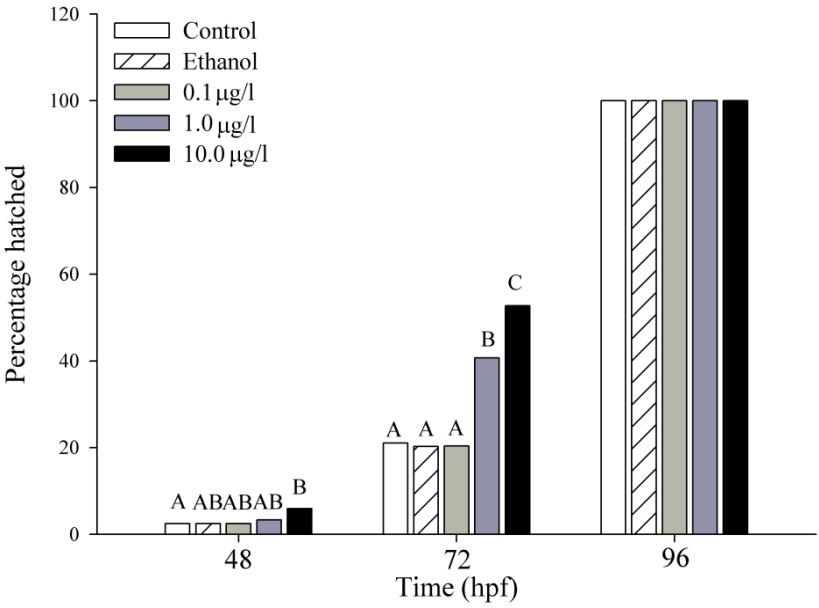


Figure 4A

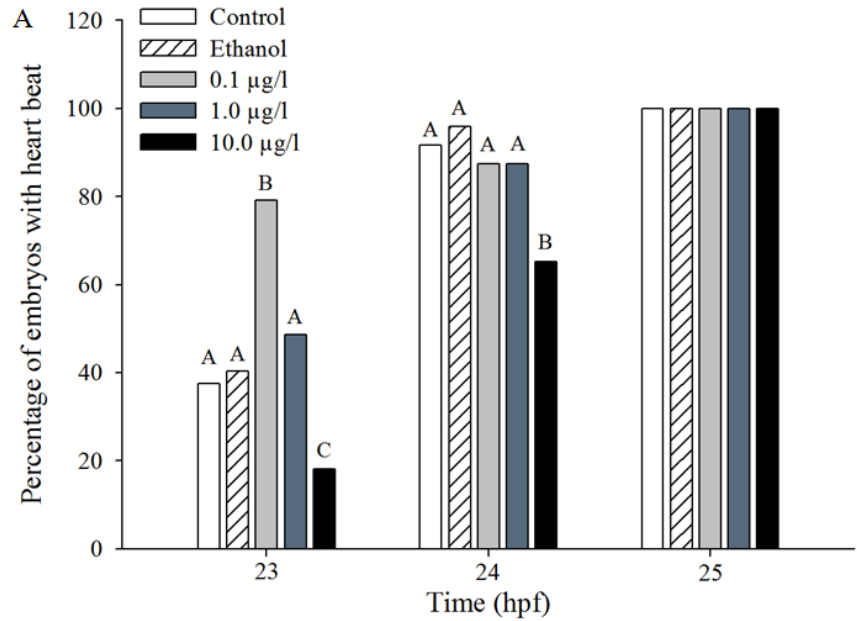


Figure 4B

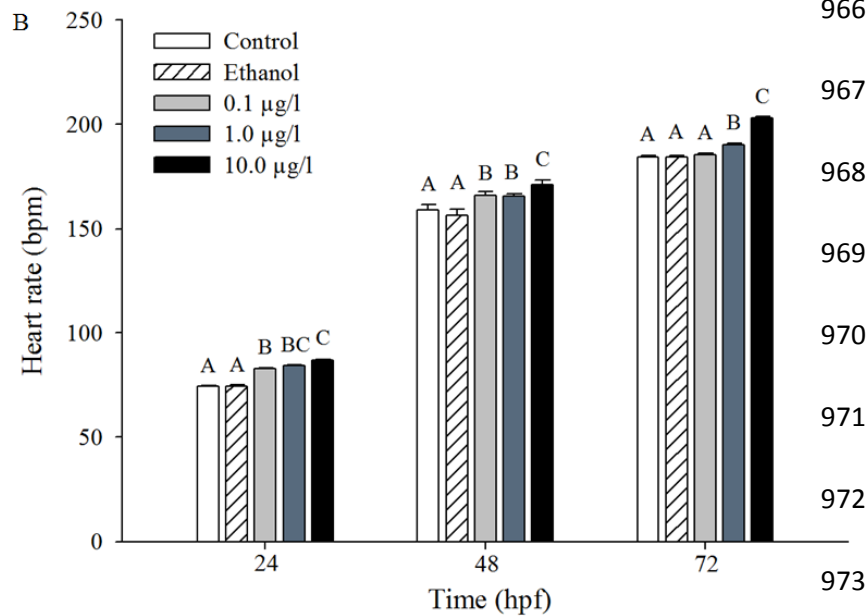


Figure 5

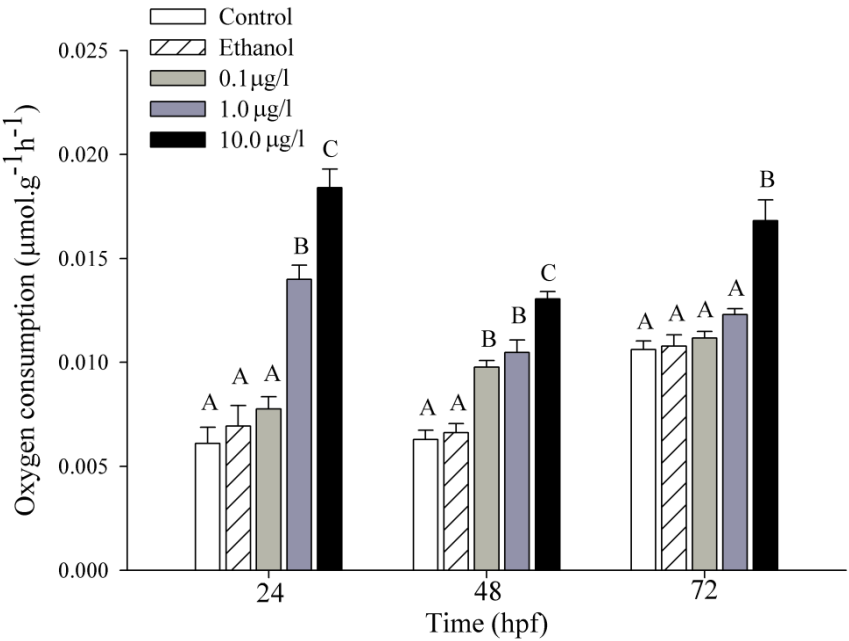


Figure 6

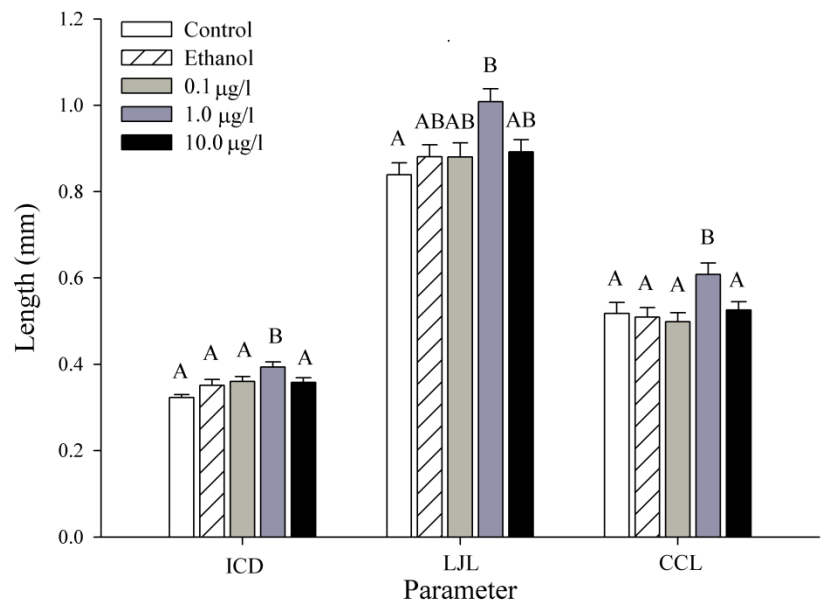


Figure 7A

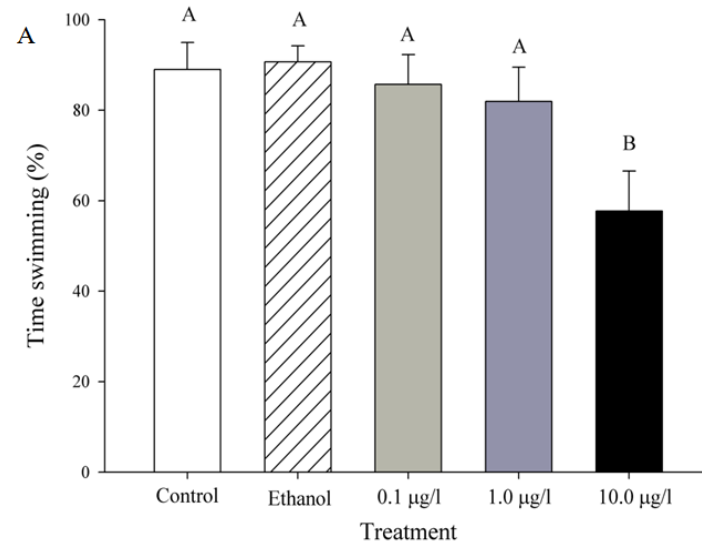


Figure 7B

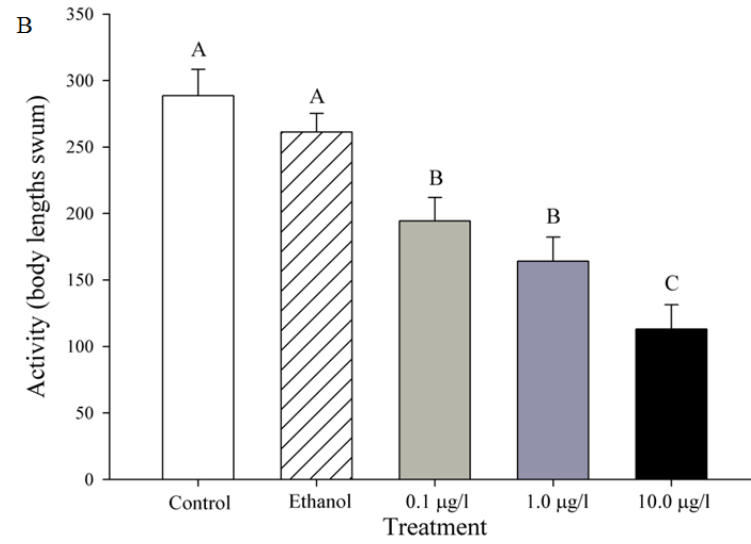


Figure 7C

